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Rapid apoplastic pH measurement in Arabidopsis leaves using a fluorescent dye

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In plants, the extracellular space (apoplast) is one of the main places where exchange of molecules occurs between cells. Not only is this compartment involved in the storage of multiple metabolites and ions, including calcium and protons, but it also plays a role in the transmission of signaling molecules for cell-to-cell communication. It has recently been shown multiple times that these two aspects are linked and can influence each other. In particular, apoplast pH was shown as a primary regulator of auxin (IAA) transport in *Arabidopsis thaliana*. To prove the role of apoplastic pH, we have developed a protocol for apoplastic fluid extraction from Arabidopsis leaves, followed by pH determination using the 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) fluorescent dye. This technique successfully allows one to monitor apoplastic pH variations among different plant lines and to link changes in apoplastic pH to cellular responses in the plant.

Keywords: pH measurement, HPTS, apoplast

Abbreviations: HPTS, 8-Hydroxypyrene-1,3,6-trisulfonic acid; IAA, indol acetic acid

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The extracellular space, also called the apoplast, has proven to be an essential storage place for several ions, including calcium or protons.[1,2] A plant's ability to tightly regulate transport across the plasma membrane is crucial to maintaining proper homeostasis of these ions as well as other molecules, such as hormones. In fact, auxin (IAA) transport is known to be highly dependent on pH, and studies of auxin-mediated plant development[3] and guard cell movement[4] have shown the effect of vacuolar proteins on IAA transport via indirect modification of apoplastic pH. In addition, apoplastic pH in leaves increases under drought conditions, which facilitates rapid ABA transport into guard cells.[5] Thus, monitoring apoplastic pH under various conditions or between different plant lines can reveal important physiological processes regulating, or under the control of, proton transport across the plasma membrane. While methods allowing one to retrieve apoplastic fluids have already been established, pH determination of such small volumes in a serial manner remains, to our knowledge, absent from the literature. To address this, we have developed a protocol for quick and easy pH measurement of small samples. Here we provide detailed procedures, which could help to monitor apoplastic pH variations in plants, thus allowing for a link between apoplastic pH and cellular response.

Apoplastic fluids are extracted using the vacuum-exfiltration method.[6] About 50 leaves from 4- to 5-wk-old plants are collected and immersed in 50 mL of water in a filtering flask sealed with a rubber cap and connected to a vacuum pump. Vacuum (100 psi) is then applied for 1 min under constant, vigorous shaking, after which the cap is forcefully removed for about 2 sec before it is put back in place to recreate vacuum conditions. This cycle is repeated 10 times, and correctly treated leaves appear darker than their untreated counterparts and tend to sink to the bottom of the flask.

The surface of the leaves is thoroughly dried using paper towels, and the leaves

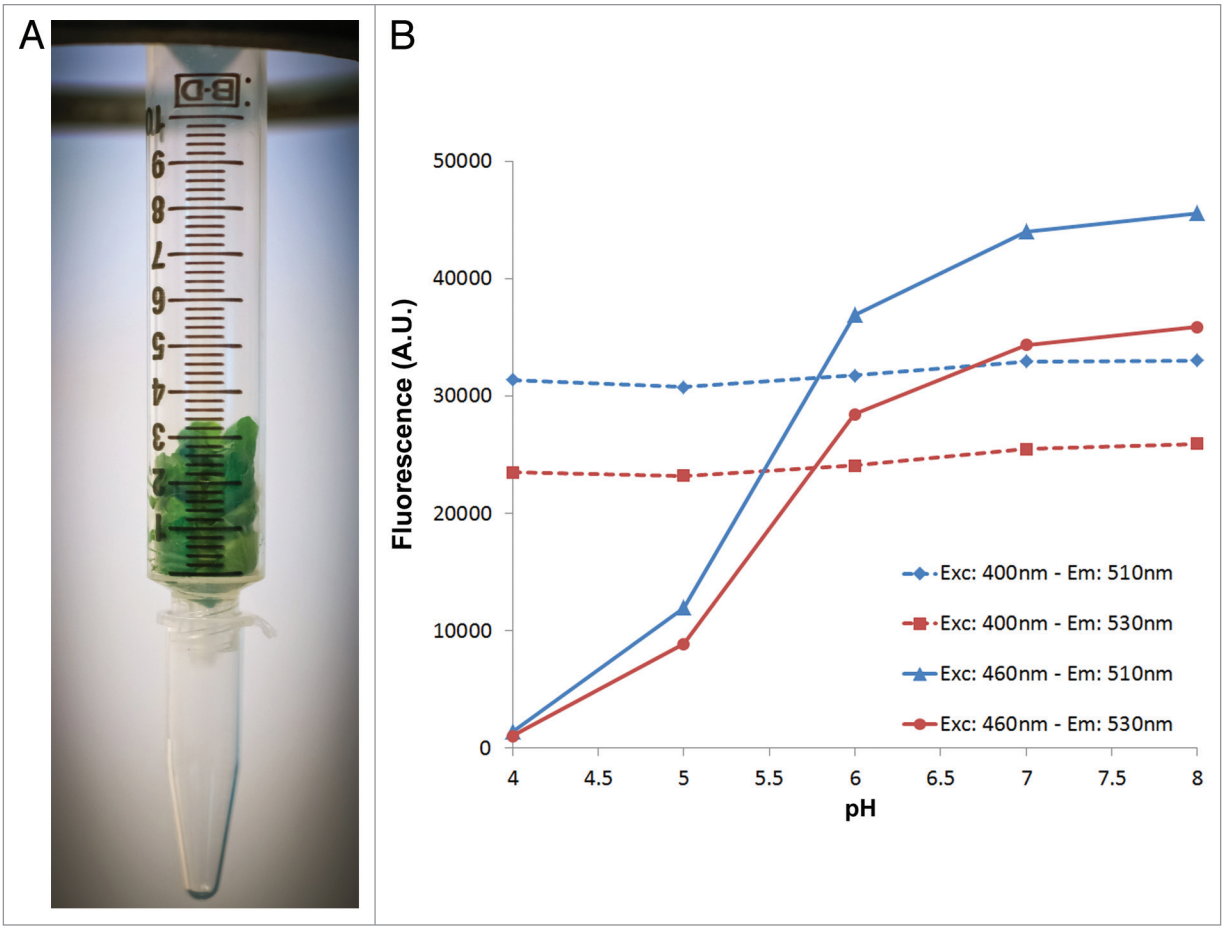

Figure 1. Apoplastic pH measurement of Arabidopsis leaves. (**A**) To collect apoplastic fluids, vacuum-treated leaves (c.a. 50 leaves) are placed inside a syringe without its plunger that is connected to a 1.5 mL centrifuge tube. (**B**) Fluorescence emission at 510 nm (blue diamond and triangle) and 530 nm (red square and circle) of the HPTS dye as a function of pH, when excited at 400 nm (dashed line) and 460 nm (solid line).

are placed in the barrel of a syringe, the plunger of which has been removed. The tip of the syringe is then inserted into a 1.5 mL centrifuge tube (**Fig. 1A**), and the whole mounting is placed inside a 50 mL conical tube. The tube is spun at 4,000 g for 15 min at 4°C, and apoplastic fluids are collected in the 1.5 mL tube. If too much debris have pelleted, the supernatant can be transferred into a new tube and kept at 4°C for a few minutes until use. Even though we have successfully measured the pH of apoplastic fluid samples following storage at -80°C, we recommend proceeding immediately to pH measurements as we noticed a slight decrease in reproducibility after 1 wk at -80°C that resulted in larger standard deviations (data not shown). It is also important to note that leaf samples prior to apoplastic fluids extraction cannot be frozen or stored at 4°C since this may cause cell lysis or possible cold-induced artifacts, respectively.[7]

HPTS is an inexpensive, non-toxic and water-soluble dye whose fluorescence is pH-dependent.[8] To evaluate the pH-dependence of HPTS, we prepared a standard curve using 40 µL of a 100 mg/mL HPTS solution (Invitrogen) in 160 µL of Britton-Robinson buffer (0.05 M H_3BO_3, 0.05 M H_3PO_4, 0.05 M CH_3COOH), whose pH ranges from 4–8 by 1-unit increments. While the manufacturer's instructions mention a shift of the excitation peak from 400–450 nm with increasing pH, we observed a rather stable fluorescence when excited at 405 nm that was poorly correlated with pH [**Fig. 1B**, dashed lines, emission collected at 510 nm (blue diamonds) and 530 nm (red squares)]. However, when excitation light was set to 460 nm (**Fig. 1B**, solid lines), the emission intensity was well-correlated with the pH of the solution [emission collected at 510 nm (blue triangles) and 530 nm (red circles)]. Interestingly, the slope of the curve is maximum between pH 4–6, allowing for accurate measurements of the pH of a solution in the physiological range of apoplastic pH.[9]

As opposed to what is described in the manufacturer's instructions, it was not feasible to use an excitation ratio of 460/405 since a slight increase in the 405 nm excitation from pH 6 to pH 8 tends to flatten the 460/405 curve after pH 6. The use of raw fluorescence values, rather than ratios, appears to make the assay more sensitive to changes in the dye concentration between technical replicates, requiring the use of a standard curve for each measurement series. We routinely make a 10-point curve ranging from pH 3.0 to pH 6.0 by 0.3-unit increments and use three technical replicates per biological sample.

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